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TITLE: **Enhancement of Vitamin D Action in Prostate Cancer through Silencing of CYP24**

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14. ABSTRACT
This study focuses on the enzyme, CYP24 which hydroxylates vitamin D acting to catalyze the first step in the breakdown of Vitamin D, effectively limiting this growth inhibitory signaling pathway. We are testing the hypothesis that hrough the inhibition of CYP24 using an siRNA approach we can convert prostate cancer cells that are resistant to the antiproliferative actions of Vitamin D to cells that are growth inhibited at low concentrations of Vitamin D. Inhibition of 1,25(OH)2D3 CYP24 mediated metabolism to potentiate Vitamin D actions in prostate cancer shows great potential for both a chemopreventative approach and the treatment of advanced hormone refractory cancer in patients. We have tested CYP24 siRNA constructs, ketoconazole and silencer control siRNA on three cell lines (LNCaP, PC3 and DU145) and evaluated CYP24 protein expression, mRNA expression, and growth inhibition. We are in the process of developing the stable transfected cell lines and optimal approach to enhance Vitamin D action in resistant cells.

15. SUBJECT TERMS
Prostate cancer, vitamin D, CYP-24 (24-hydroxylase), growth inhibition, apoptosis

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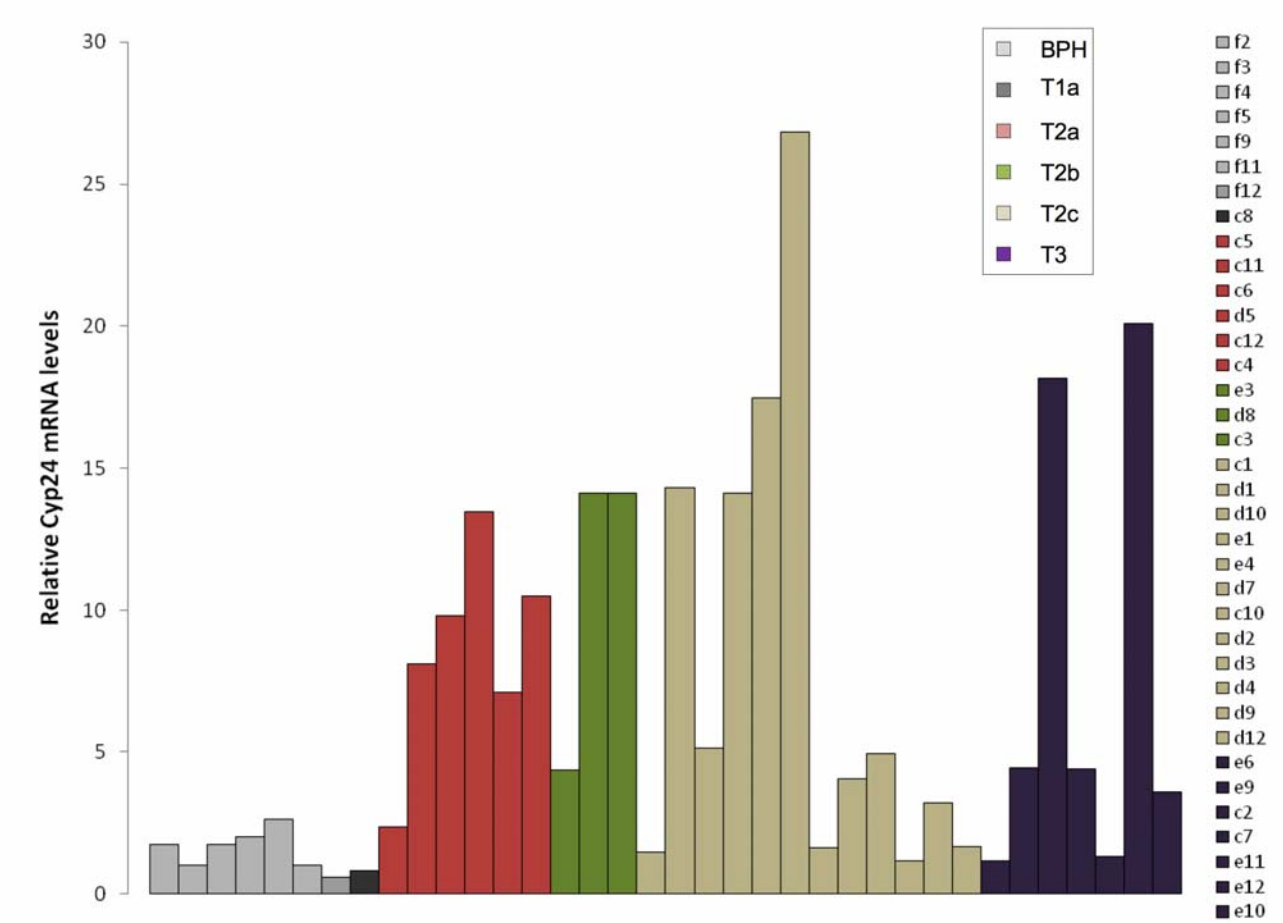

Figure 1 Profiling of Cyp24 gene expression levels across human prostate cancer progression stages. TaqMan QPCR was performed using specific Cyp 24 primers. B actin was used as an internal control. Patients' cDNA were purchased from Origene (www.origene.com) and were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels.

Many prostate tumors exhibit increased expression of CYP24A1, effectively limiting biologic activity of the vitamin through enhanced metabolism. This study focuses on approaches inhibit CYP24A1 in prostate cancer cells. Using Q-PCR we have performed a more in depth analysis of the incidence of CYP24 over-expression at different stages of prostate cancer (Figure 1). As can be seen above, expression of CYP24 is low in

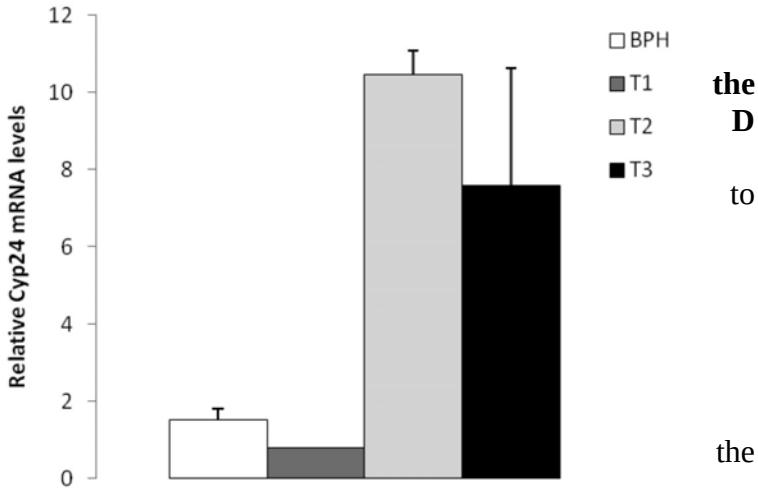

Figure 2 Gene expression analysis of Cyp24 in human patients with various TNM stages of prostate cancer (Same data as previous)

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BPH and markedly increased in TMN stages T2a, T2b, T2c and T3, although expression was not elevated in every single sample. Taken together, Figure 2 represents this data as the mean values by stage and the study again shows that there is a marked elevation of CYP24 with increasing stage, although with greater heterogeneity at T3.

Similarly, the expression of CYP24 was increased in the LNCaP and PC-3 cell lines (as well as DU145, not shown). Levels were undetectable in LNCaP cells and low in the androgen resistant PC-3 cell lines cultured in the absence of vitamin D. Notably, when comparing LNCaP and PC-3 cells differences in the relative level of expression of CYP24 varied 10 fold after 48 hr culture in the presence of 10 nM Vitamin D, an agent known to induce CYP24 expression levels in non-

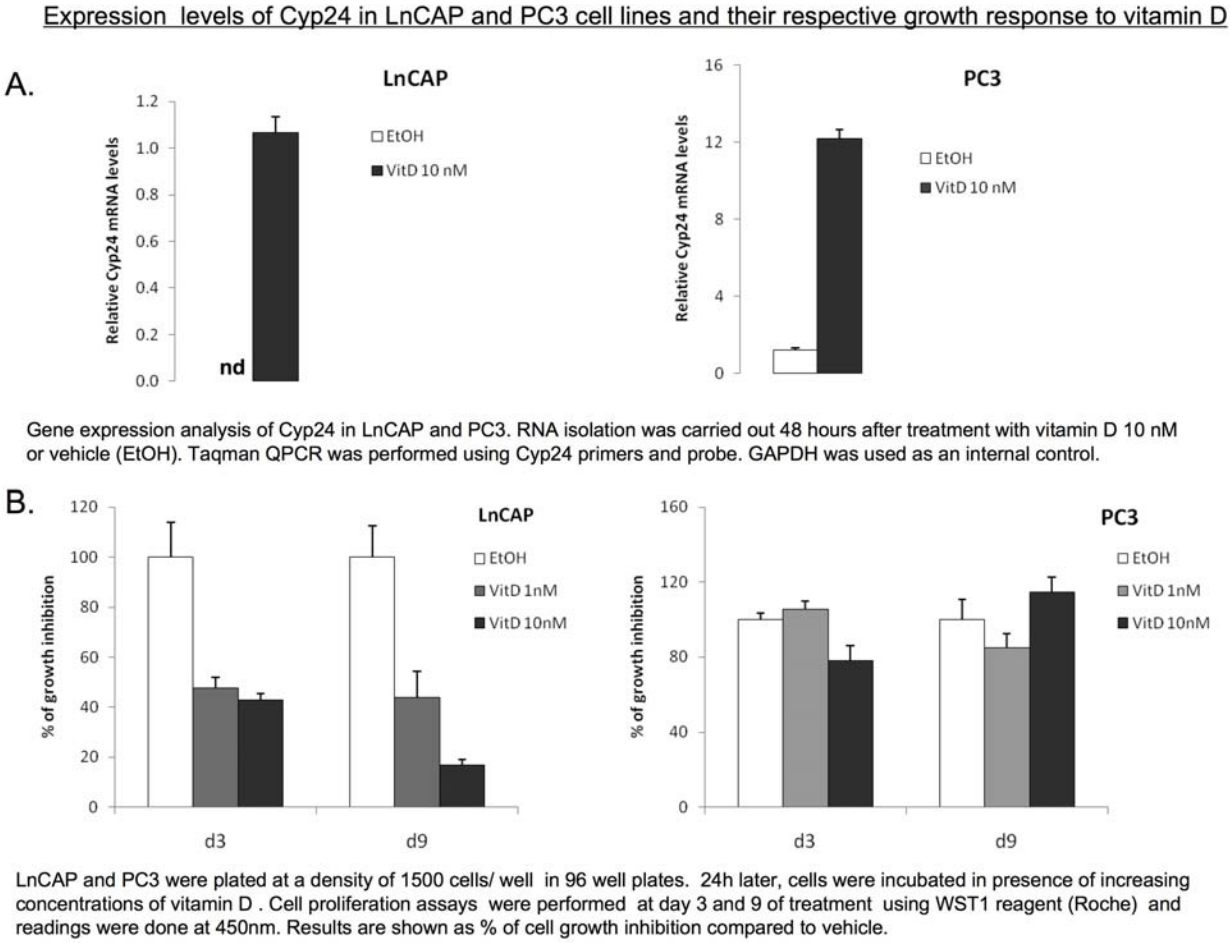

Figure 3 Vitamin D administration regulates CYP24 gene expression in LNCaP and PC-3 prostate cancer cell lines. The proliferation of LnCAP cells is inhibited by increasing concentration of vitamin D, while PC-3 cell proliferation is largely unaffected.

malignant Vitamin D responsive cells.

<u>Specific Aim #1</u> ***To enhance Vitamin D inhibition of prostate cancer growth through inhibition of CYP24.***

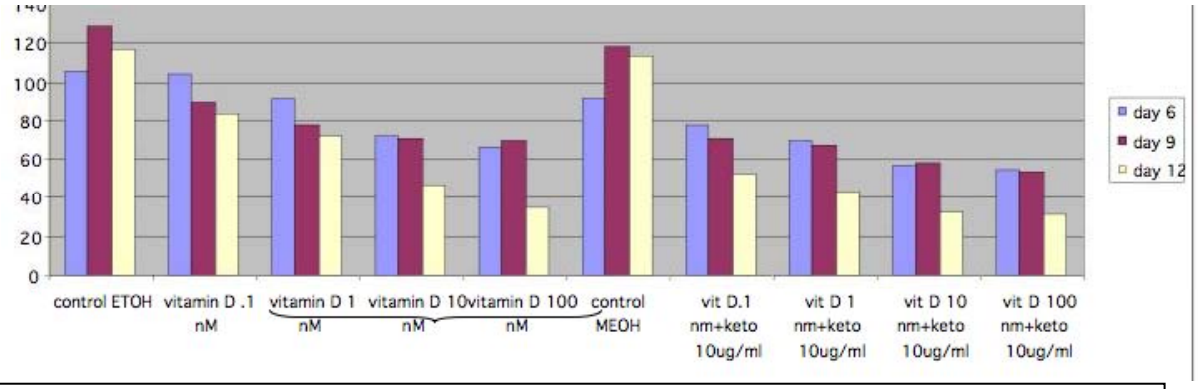

Figure 4 The Antiproliferative Effect of Low Dose Vitamin D Is More Potent In The Presence of Ketoconazole

Aim #1 has been largely completed and a manuscript is in preparation. We tested the hypothesis that inhibition of CYP2A14 could enhance the antiproliferative actions of Vitamin D in prostate cancer. LNCaP cells were cultured in the presence or absence of Vitamin D at increasing concentrations and increasing concentrations of ketoconazole over a 9-day period with cell counts on days 0, 3, 6, 9, and 12. Each treatment was performed in triplicate with duplicate cell counts. Under these conditions, for the LNCaP cell line, administration of Vitamin D3 resulted in a significant dose dependent inhibition of cell proliferation and administration of Vitamin D together with ketoconazole showed enhanced inhibition at lower concentrations of Vitamin D (Figure 4). Despite the broad effects of ketoconazole, results suggested that inhibition of CyP24 (one known actions of ketoconazole) could enhance Vitamin D action. This observation served as the positive control for this study. In the Preliminary Studies presented, we had tested a small collection of CYP24A1 siRNAs to knock down CYP24 protein levels in prostate cancer cell lines. A much more complex pool of CYP24A1 siRNAs effectively diminished CYP24A1 levels in the three cell lines tested (LNCaP, PC-3 and DU145) (Figures 2,e). In Figure 5, we show that pooled siRNA inhibited CYP24A1 expression in the PC-3 and DU145 cell lines as compared with the controls treated with scrambled non-specific siRNA. Under these culture conditions in minimal culture medium, the CYP24 levels were undetectable under basal conditions. A positive regulator of CYP24A1 gene expression is Vitamin D, which enhances CYP24A1 gene

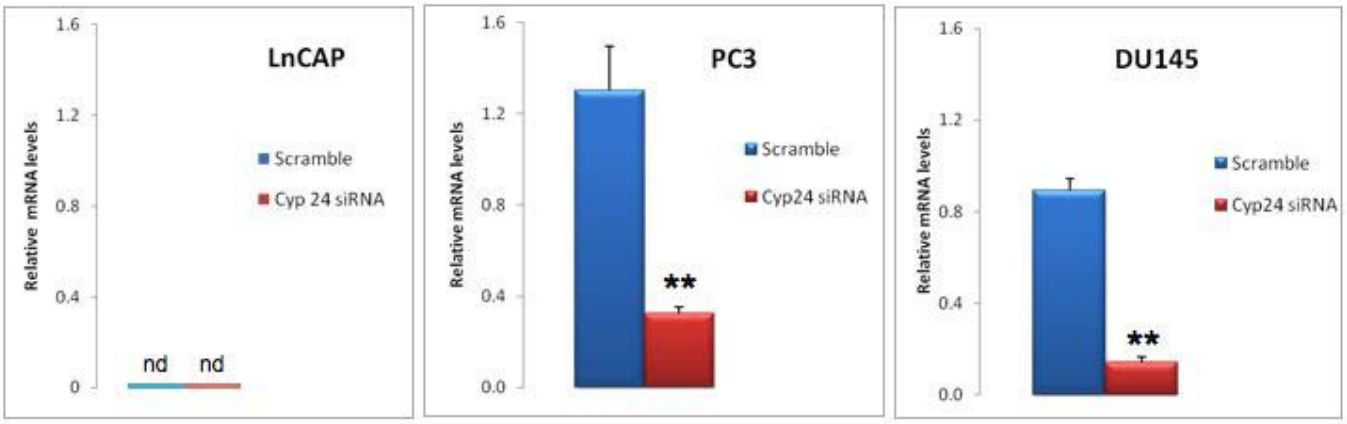

Figure 5 Knockdown of CYP24 Expression in Human Prostate Cancer Cell Lines Using Pooled CYP24 siRNA

expression. Figure 6 shows that the pooled siRNA also effectively inhibited the induced, higher levels of CYP24A1 observed after treatment with 10 nM Vitamin D for 48 hr. This inhibition is seen at the protein level as well as reflected in the immunocytochemical analysis of CYP24 protein in the presence or absence of pooled siRNA shown in Figure 7. In the presence of Vitamin D (which induces CYP24 expression), several cells appear to have not effectively taken up the siRNA while the majority shows nearly complete inhibition at the protein level.

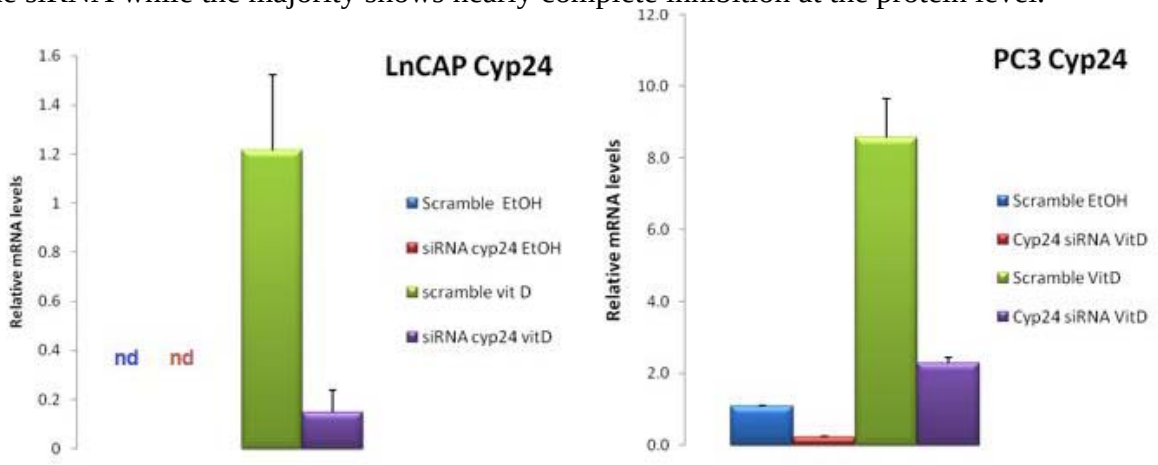

Figure 6 Gene expression analysis of Cyp24 in CAP and PC3 transiently transfected with Cyp 24 siRNA. Taqman QPCR used sequence-specific primers and probe. GAPDH was the internal control. RNA was isolated 48 hours after treat ment

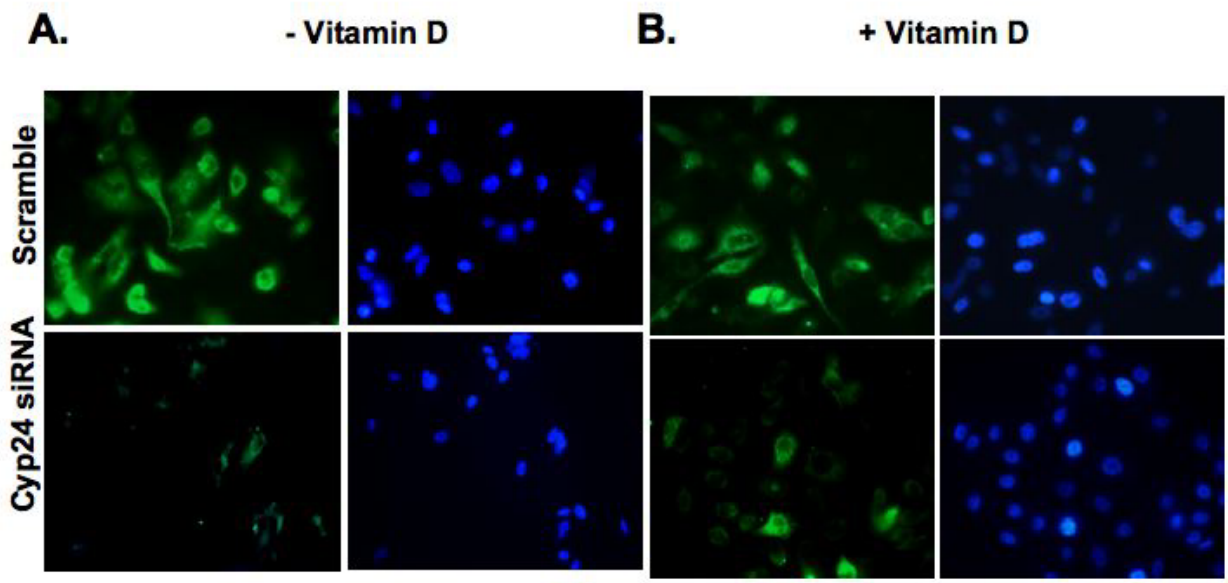

Figure 7. CYP24 siRNA inhibits CYP24 basal and vitamin D induced expression of CYP24 in cultured PC-3 cells revealed by immunocytochemistry. Under the vitamin D induced levels, some isolated cells escaped the knockdown. Results show efficient knockdown of CYP24 protein

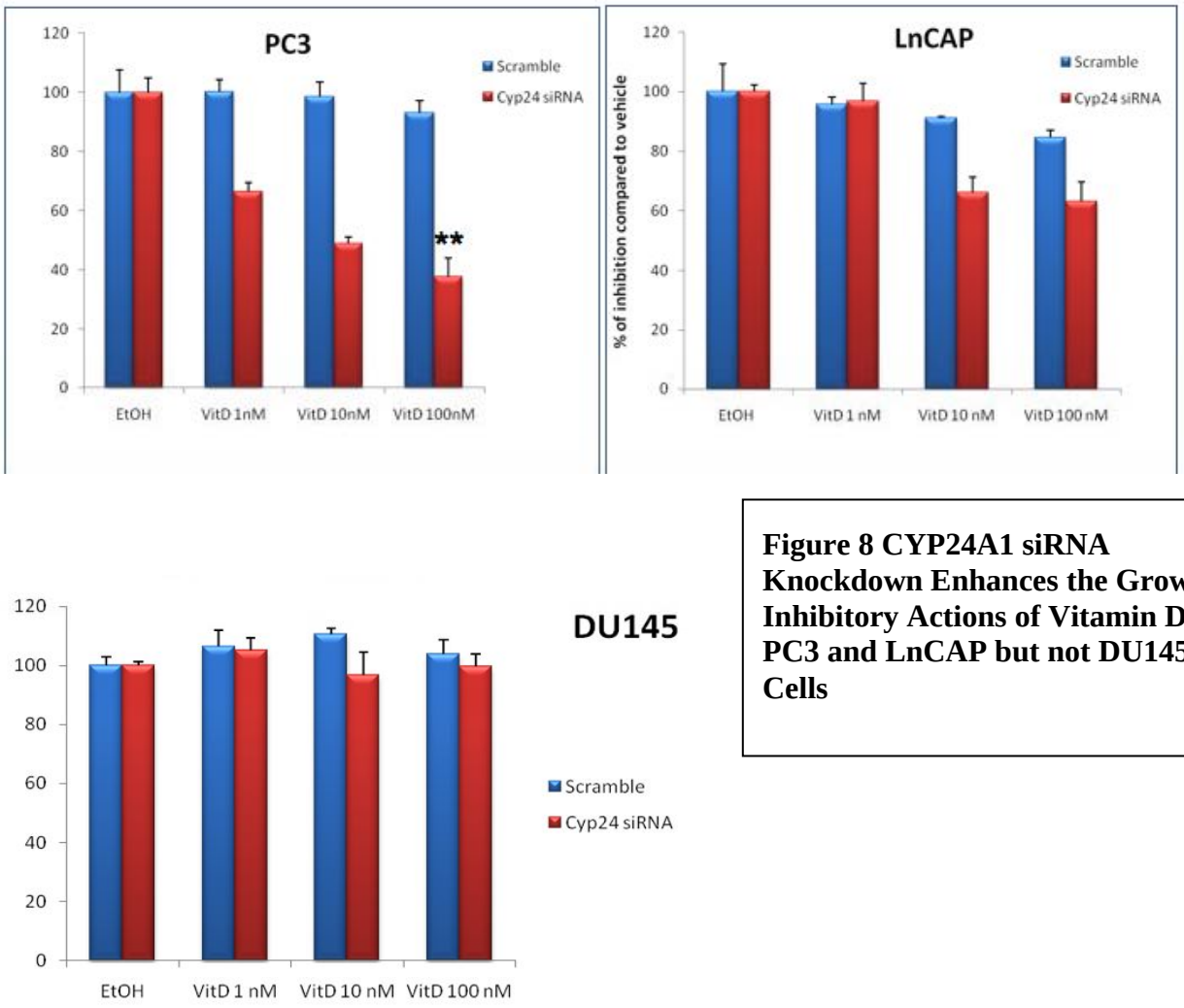

Figure 8 CYP24A1 siRNA Knockdown Enhances the Growth Inhibitory Actions of Vitamin D in PC3 and LnCAP but not DU145 Cells

This knockdown also enhanced the anti-proliferative activities of Vitamin D in PC-3 and LNCaP cells. As shown in Figure 8, low dose Vitamin D administration was far more growth inhibitory when pooled CYP24A1 siRNA inhibited CYP24A1 in the cells. CYP24siRNA-PC-3 cells were much more growth inhibited by Vitamin D administration, then the scrambled siRNA controls. This was true for the LNCaP cells but to a lesser extent. As expected, knockdown of the CYP24A1 in DU145 cells did not cause the cells to change from Vitamin D insensitive to Vitamin D responsive because this cell line lacks P53 as has been observed earlier by Weigel and colleagues.

Knockdown of CYP24A1 enhanced the expression of genes known to be regulated by Vitamin D. Expression of p21 (ligand-Vitamin D receptor induced transcription) was enhanced by CPY24 knockdown and repression of Cyclin D1 was slightly increased by CYP24 knockdown. (Figure 10). Similarly, transient transfection with VDRE-tk-luc and treatment with pooled CYP24A1 enhanced Vitamin D induced transcriptional activity. As shown in Figure 9, Vitamin induction of luciferase expression was modest with treatment with control-scrambled siRNA, whereas induction was potentiated in the presence of CYP24A1 siRNA.

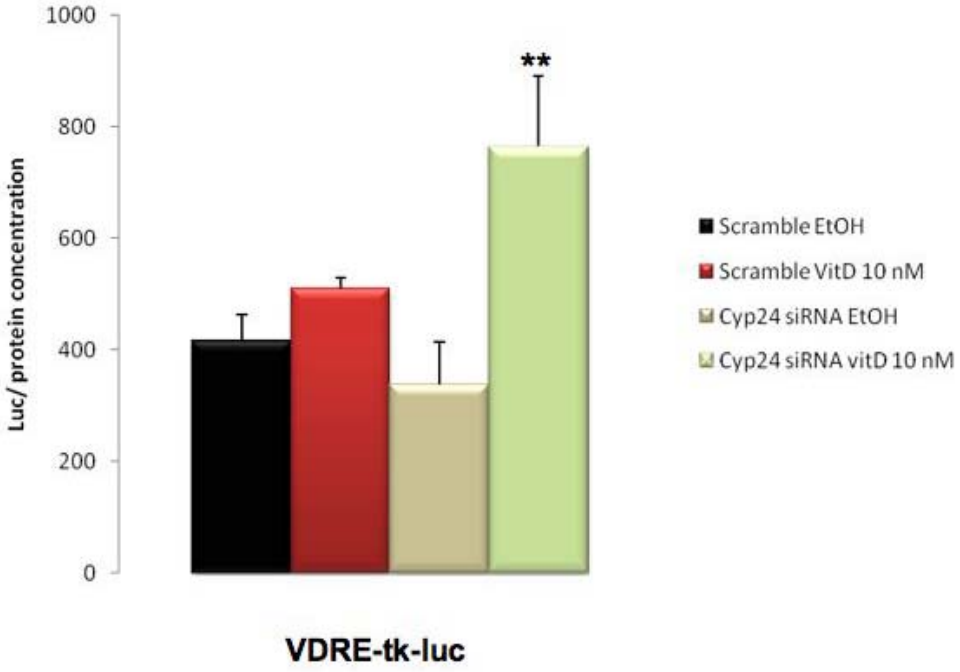

Figure 9 Transcriptional activity of vitamin D is enhanced in presence of Cyp 24 siRNA

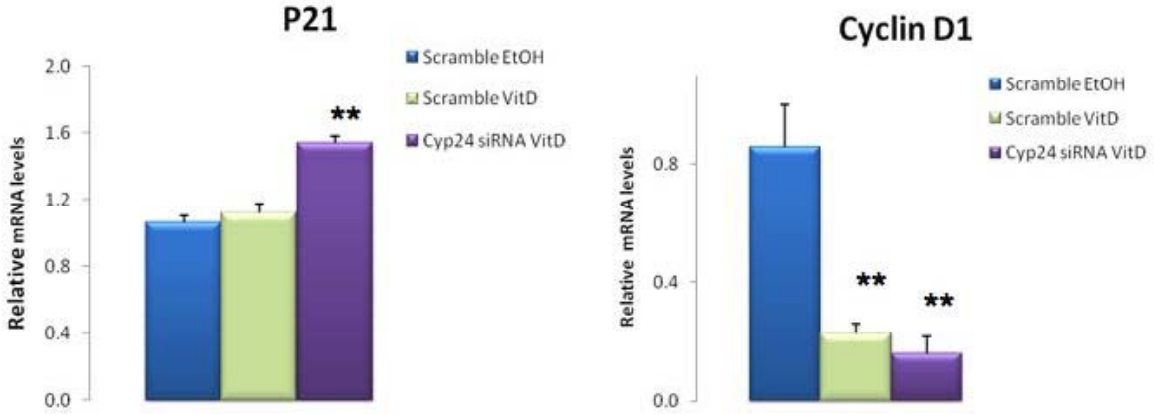

Figure 10 Regulation of Cell Proliferation Marker Gene Expression In Presence Of Cyp 24 siRNA And Vitamin D In PC3

<u>Specific Aim #2</u> To Enhance Vitamin D inhibition of prostate cancer proliferation by CYP24 siRNA in vivo.

To examine the efficacy of CYP24 knockdown on prostate cancer growth in vivo, it was necessary for us to create stable cell lines expressing CYP24 shRNA. We used lentiviral particles containing Cyp24 shRNA and stable cell lines were selected with puromycin. Non-targeting particles were used as control (scramble). As shown in Figure 11 stable cell lines were created for PC-3 and LNCaP cells that displayed CYP24 knockdown in the presence or absence of vitamin D administration and enhanced growth inhibition (panel B) and inhibition of cyclin D1 mRNA expression (panel C). We have begun xenograft studies to examine the efficacy of vitamin D administration in vivo for growth inhibition prostate cancer cells.

Figure 11 Stable CYP24A1 Expressing shRNA Cell Lines